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Unfolding Ubiquitin by force: water mediated H-bond destabilization

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Abstract. Using the "pull and wait" (PNW) simulation protocol at 300 K, we studied the unfolding of a ubiquitin molecule by force. PNW was implemented in the CHARMM program using an integration time step of 1 fs and a uniform dielectric constant of 1. The ubiquitin molecule, initially solvated, was put under mechanical stress, exerting forces from different directions. The rupture of five hydrogen bonds between parallel strands β1 and β5 takes place during the extension from 13 to 15 Å, defines a mechanical barrier for unfolding and dominates the point of maximum unfolding force. The simulations described here show that given adequate time, a small applied force can destabilize those five H-bonds relative to the bonds that can be created to water molecules; allowing the formation of stable H-bonds between a single water molecule and the donor and acceptor groups of the interstrand H-bonds. Thus, simulations run with PNW show that the force is not responsible for "ripping apart" the backbone H-bonds; it merely destabilizes them making them less stable than the H-bonds they can make with water. Additional simulations show that the force necessary to destabilize the H-bonds and allow them to be replaced by H-bonds to water molecules depends strongly on the pulling direction. By using a simulation protocol that allows equilibration at each extension we have been able to observe the details of the events leading to the unfolding of ubiquitin by mechanical force.

Key words: H-bond, molecular dynamics, PNW, mechanical unfolding

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Desplegamiento por fuerzas de ubiquitina: desestabilización de enlaces de hidrogeno mediado por agua. Resumen. Estudiamos el desdoblamiento forzado de una molécula de ubiquitina, usando el protocolo de dinámica molecular "pull and wait" (PNW) a 300 K. Se implementó PNW en el programa CHARMM usando un tiempo de integración de 1 fs y una constante dieléctrica de 1. La estructura solvatada inicialmente, se colocó bajo tensión mecánica, ejerciéndose fuerzas en diferentes direcciones. El rompimiento de cinco enlaces de Hidrogeno entre los pliegues β1 y β5 que tiene lugar durante los primeros 13 a 15 Å de extensión, marcan una barrera mecánica la cual define la máxima fuerza necesaria para el desdoblamiento. Las simulaciones realizadas muestran que dado un tiempo adecuado, la aplicación de una fuerza pequeña puede desestabilizar los mencionados enlaces de hidrógeno relativo a los enlaces que se pueden formar con moléculas de agua; permitiendo la formación de enlaces de hidrógeno estables entre aguas y donadores o aceptores de la cadena principal. De esta forma, las simulaciones con PNW muestran que la tensión mecánica no es responsable de separar los puentes de hidrógeno, esta sólo los desestabiliza haciéndolos menos estables con respecto a los enlaces que se pueden formar con moléculas de agua. Simulaciones adicionales muestran que la fuerza necesaria para desestabilizar los enlaces de hidrogeno, permitiendo su remplazo por enlaces con moléculas de agua, depende fuertemente de la dirección de estiramiento. El protocolo de simulación que permite equilibrar a cada paso de extensión, nos evidenció eventos conducentes al desdoblamiento de la molécula ubiquitina por fuerzas mecánicas.

Resumo. Estudamos o desdobramento forçado de uma molécula de ubiquitina usando o protocolo de dinâmica molecular "pull and wait" (PNW) em 300 K. PNW foi implementado no programa CHARMM usando um tempo de integração de 1 fs e uma constante dielétrica de 1. A estrutura solvatada, foi colocada sob estresse mecânico, exercendo-se forças de diferentes direções. Simulações mostraram que a ruptura de cinco ligações de Hidrogênio entre as dobras β1 e β5, que acontece durante os primeiros 13 a 15 Å de extensão, define uma barreira, a qual determina a força máxima para o desdobramento. As simulações mostram que, dado o tempo adequado, uma pequena força aplicada pode desestabilizar os mesmos cinco Hidrogênios relativos às ligações H- que os grupos da cadeia principal podem fazer com a molécula de água; permitindo a formação de ligações estáveis de H- entre moléculas de água e os grupos doadores e receptores da intercadeia. Desta forma, simulações que utilizam este protocolo mostram que a força não é utilizada para «romper» as ligações H- da cadeia principal, apenas desestabilizando-as e tornando-as menos estáveis do que as ligações que as mesmas podem fazer com a água. Simulações adicionais mostram que a força necessária para desestabilizar as ligações H- e permitir que as mesmas sejam substituídas por ligações do Hidrogênio com moléculas de água depende fortemente da direção da aplicação da força. Através da utilização de um protocolo de simulação que permite equilibrar em cada extensã, fomos capazes de observar os detalhes do mecanismo de desdobramento da ubiquitina por força mecânica.

Introduction

In addition to their interactions with other molecules in the cell, biological macromolecules are subjected to mechanical forces both, functional (e.g., within muscle fibers, microtubules, and molecular motors) and incidental. Thus, as part of the optimization of their specific biological function, evolution of these molecules involves adaptation of their mechanical behavior. A recent example is the direct relation between the DNA gyrase functional mode of action and the applied mechanical stress (1).

Advances in single-molecule atomic force microscopy (AFM) and optical tweezers techniques have made possible the examination of the response of proteins to mechanical forces (2-4). The muscle protein titin, for instance, has been extensively investigated using both atomic force microscopy (AFM) methods (5-7) and optical tweezers (8, 9). Similar studies have also been conducted on T4 lysozyme (10), bacteriorhodopsin (11-13), a Na^+/H^+ antiporter (14), and other proteins (15). Unfolding forces in different pulling directions have been measured for green fluorescent protein (GFP) (16), ubiquitin (17), and the lipoyl domain (E2lip3) of the acetyl transferase subunit E2p (17). Significant differences were observed between the responses of the same molecule to pulling along different directions, reflecting path-dependent processes, and nonequilibrium events, each with their own characteristic force barriers (18).

AFM studies measuring the mechanical stability of ubiquitin monomers under various stretching conditions demonstrated the existence of well defined stages in ubiquitin folding (19-21). Carrion- Vazquez *et al.* (17) used AFM experiments and steered molecular dynamics (SMD) calculations to study the mechanical response of linked polyubiquitin chains C to N-terminus or Lys 48 to C-terminus. They found that the force required to unfold ubiquitin is strongly dependent on the direction along which the force is applied. Particularly the C-terminus to N-terminus linked polyubiquitin chains (C-to-N polyubiquitin) yielded a titin-like sawtooth pattern of unfolding forces of about 200 pN separated by 24 nm, the contour length of one ubiquitin monomer.

On the other hand, C- terminal to- Lys 48 linked polyubiquitin yielded a different saw tooth pattern with unfolding forces of about 85 pN separated by only 7.8 nm, the contour length increment that was consistent with the unfolding of a portion of one ubiquitin monomer from Lys 48 to the C-terminus.

SMD simulations (17) qualitatively reproduced the differences between these mechanical unfolding patterns and additionally showed, that the major unfolding barrier of extension by force for both C-to-N linked and C-to-Lys 48 linked ubiquitin was formed by the rupture of hydrogen bonds between specific β-strands: two parallel β-strands (I and V) in C-to-N linked ubiquitin and two antiparallel β-strands (III and V) in C-to-Lys 48 linked ubiquitins. Even though the same number of hydrogen bonds was broken in each unfolding scenario, the forces required to unfold the domains were significantly different. These results show that not only the number and nature of intra strand hydrogen bonds contribute to the resistance of β-sheets to disruption by force, but also the orientation of the strands relative to the pulling direction.

Monte Carlo simulations have also been employed to probe mechanical unfolding, normally to test relatively coarse-grained kinetic models (22-24). In particular, Szymczak and Cieplak have used a Go-type model to examine the statistics of mechanical unfolding of ubiquitin and integrin (25). Paci *et al.* (26) researched the mechanical unfolding of a number of proteins using molecular dynamics (MD) simulations, Go-type models, and other methods, to examine energetic and mechanical properties of the molecules such as resistance to force (27).

Here we studied the unfolding of ubiquitin by force (**Figure 1**), using all atom MD simulations with an alternative, speed-independent simulation protocol, the "pull and wait" technique (PNW) (21, 28).

As part of this study, we explored the mechanism of hydrogen bond-breaking events and in particular the role of water molecules in this process. Although computationally SMD and PNW are similar methods, in SMD the system is always "catching-up" with the moving tether and never equilibrates with a given position of the tether except at the very end and only if the system is allowed to equilibrate. In PNW on the other hand, even at overall speeds similar to

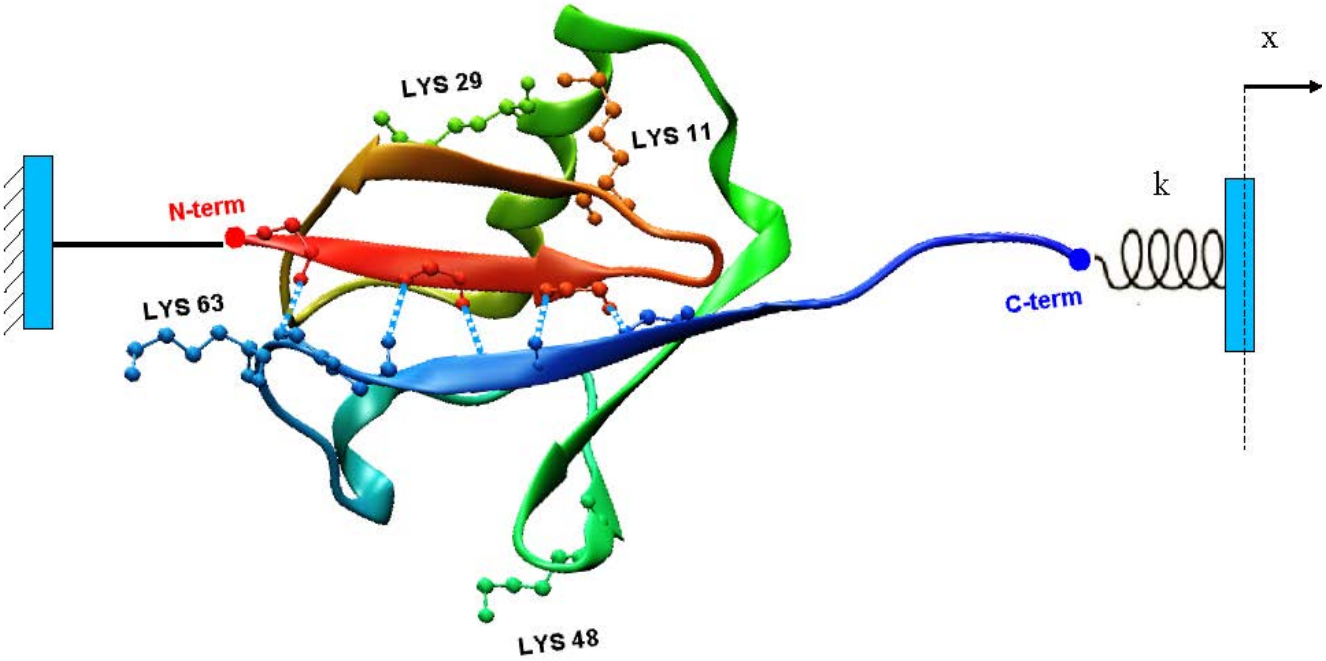

Fig. 1. Ribbon diagram showing the α/β structure of ubiquitin and CPK representation of exposed LYS residues. Native ubiquitin has a five stranded β-sheet; a 3.5 turn α-helix, and a 310 helix. The β content of 31.6 % is about twice as high as the α content. The backbone hydrogen bond between the I and V β-strands may form the first point of mechanical resistance when subjected to a stretching force between the N- and the C-termini.

those used in SMD, enough time is given at each extension to allow the system to equilibrate. All observations leading to mechanistic insight are carried out during these equilibration periods.

Methods

PNW (21, 28) was implemented in the CHARMM program (29) using an integration time step of 1 fs and a uniform dielectric constant of 1. The non-bonded coulomb and vdW interactions were calculated with a cutoff using a switching function starting at a distance of 10 Å and reaching zero at 13 Å. The x ray structure of ubiquitin (PDB accession code 1UBQ) (30) was placed in a box of TIP3 water molecules (31) the dimensions of which depended on the geometry of the pulling process: 1) for stretching from the C- and the N- terminis 68.2 x 40.4 x 40.4 Å containing 3280 water molecules; 2) for stretching from the N-terminus and Lys63 49.6 x 40.3 x 49**.** 6 Å containing 2898 water molecules; and 3) from Lys48 and the C-terminus 65.1 x 40.3 x 46.5 Å with 3659 water molecules. Periodic boundary conditions were used and no counterions were included in the calculations.

For the pulling simulations, one end was fixed and force was applied to the other end according to: 1) the Cα of the C-terminus and applying an external force to the Cα of Lys 63 or that of the N-terminus, 2) fixing the Cα of Lys 48 and applying an external force to the Cα of the C-terminus. Extension was accomplished by harmonically restraining (elastic constants of 2, 4 and 5 kcal/mol Å^2) the pulled end to a fixed restraint point positioned along the line joining the two Cα atoms. Following each extension the system was allowed to relax for 30–50 ps. After each equilibration the simulation was followed for times ranging from 100 to 200 ps. Average forces and extensions were calculated without including the relaxation period. At the completion of each run, the restraint point was moved in one step by an additional 2 or 4 Å along the same line and the simulations repeated using the same protocol. The boxes used were large enough to accommodate the molecule at the maximum extension used in each study without crossing the box boundary. PNW simulations were performed at 300 K, the energy of the hydrogen bond is implicitly included in the CHARMM22 force field by using the appropriate

parameterization of the partial charges and vdW parameters (32).

Even though the effective pulling rates may be similar to those used in SMD calculations, in PNW after a fast extension the system is allowed to equilibrate, allowing the dynamics run to sample events that take place during extension.

Results

Stretching ubiquitin between the C- and the N- termini: PNW was applied by fixing the Cα of the C-terminus and applying a harmonic potential (elastic constant of 3 kcal /mol Å^2) to the Cα of the N-terminus. The system contained 11071 atoms including 9840 from 3280 TIP3 water molecules. The simulation time after each 2 Å step was 200 ps. The force extension curve (**Figure 2**) shows a peak of 1450 pN at an extension of ~15 Å. In addition, the pulling process was repeated with elastic constant of 2, 4 and 5 kcal/mol Å^2. The maximal forces reached in these simulations were between 1320 and 1450 pN.

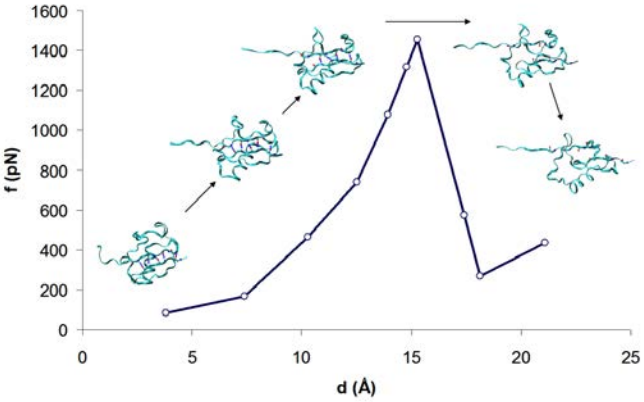

Fig. 2. Force-extension profile resulting from PNW simulations (4 Å per step) and snapshots of key events. When the two β-strands are pulled along the parallel direction, breaking of hydrogen bonds between strands β1 and β5 forms the barrier to the mechanical unfolding and dominates the maximum unfolding force.

The rupture of five hydrogens bonds (distance C_O-N_H> 3.5 Å) between parallel strands β1 and β5 takes place during the extension from 13 to 15 Å (**Figure 3**). Breaking of these hydrogen bonds defines a mechanical

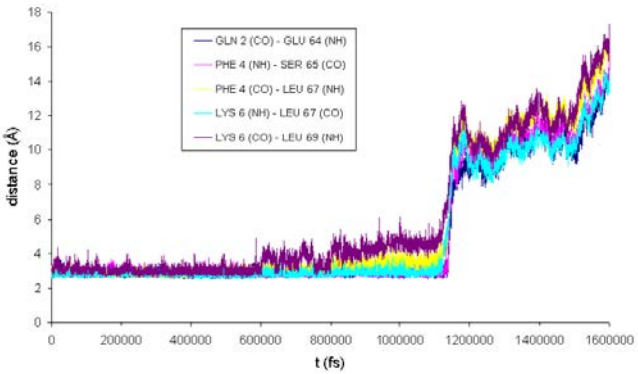

Fig. 3. Distances between H-bond donor and acceptor of β1 - β5 during the simulations.

To evaluate the effect of simulation time on the force required for H-bond breaking, simulations for two of the runs were extended to 10 ns (set A **Figure 4** and set B **Figure 5**) (21) starting at the points corresponding to the two extensions preceding the point of maximal force. No H-bond breaking occurred during 200 ps of simulation, as a result of the extension. When the simulation was extended for set A, H-bond breaking events (distance C_O-N_H> 3.5 Å) were registered at 400-500 ps with forces between 900 and 1000 pN (Figure 4).

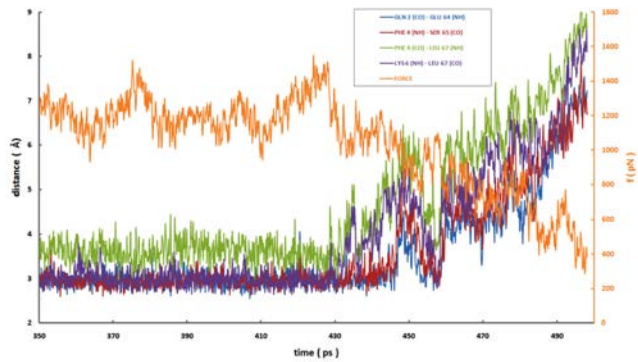

Fig. 4. Distance between H-bond donors and acceptors of strands β1-β5 of ubiquitin during the extended simulations of set A. The elastic force is shown in orange.

During this process, some H-bonds broke for short periods of time (0.1-0.3 ps) but they remained broken only when water molecules made H-bonds to the donor and acceptor groups of the protein.

In the 10-ns simulations carried out for set B, H-bond breaking took place with forces ranging between 600-700 pN (**Figure 5**).

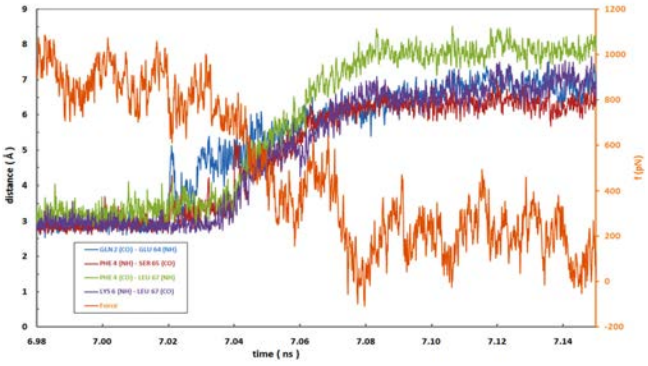

Fig. 5. Distance between H-bond donors and acceptors of strands β1-β5 of ubiquitin during the extended simulations of set B. The elastic force is shown in orange.

To rule out the possibility that even in the absence of a force some interstrand H-bonds may break in a long time simulation, the initial unextended structure was subjected to a 10-ns simulation with the two ends fixed at their original positions. Although interstrand H-bonds broke and reformed in this run, none remained broken for an extended period of time (<0.3 ps).

Stretching ubiquitin between C-terminus and Lys48: The Cα of Lys 48 was fixed and the Cα of the C terminus was pulled with an external force (elastic constant of 2 kcal/mol Å2) along the Cα to Cα direction (**Figure 6**), using one hundred ps of simulation and 2 Å of extension per step.

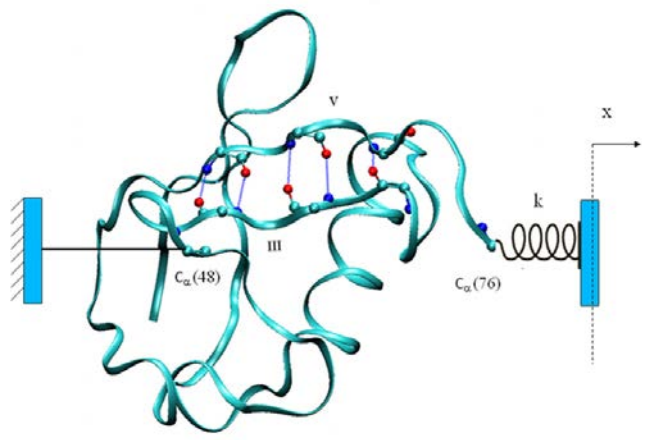

Fig. 6. PNW simulation scheme. The Cα of Lys48 was fixed and a harmonic potential was applied to the Cα of the C-terminal. The backbone hydrogen bond between strands β3 and β5 may form the first point of mechanical resistance when exposed to a stretching force between such points.

The force extension curve (**Figure 7**) shows a set of consecutive peaks where each peak is associated with a single H-bond breaking event. The simulation was terminated when a total extension of 40 Å was reached.

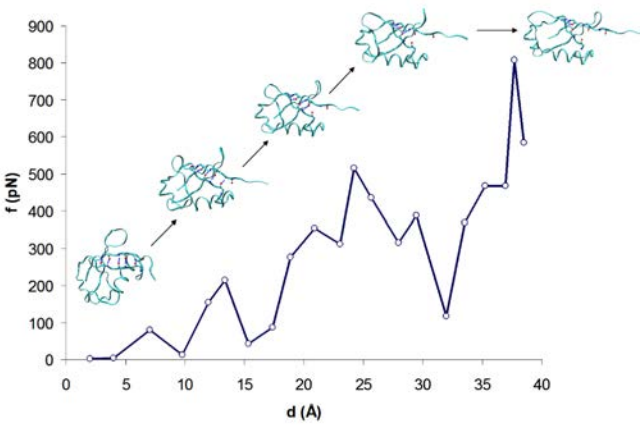

Fig. 7. Profile of force-extension between the C-terminus and Lys 48 from PNW simulations (2 Å per step) and snapshots of key events. When the end of the box was reached the simulations was terminated. Only three of five hydrogen bonds between strands β1 and β5 were broken during this process.

No H-bond breaking occurred during the initial 900 ps of simulation. After that time, three H-bonds Gln 40 (CO) – Arg 72 (NH), Arg 42 (NH) – Val 70 (CO) and Arg 42 (CO) – Val 70 (NH) broke with forces of ~200, ~500 and ~800 pN respectively (**Figure 8**).

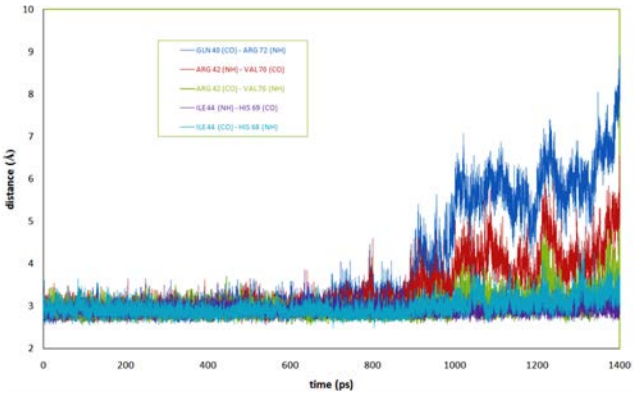

Fig. 8. Distances between H-bond donor and acceptor of β3 - β5 during the simulations. During the initial 900 ps of simulation no H-bond breaking occurred. After that time, three H-bonds Gln 40 (CO) – Arg 72 (NH), Arg 42 (NH) – Val 70 (CO) and Arg 42 (CO) – Val 70 (NH) broke with forces of ~200, ~500 and ~800 pN respectively.

Stretching ubiquitin between its N-terminus and Lys63: PNW simulations were also implemented by fixing the Cα of Lys 63 and pulling the Cα of the N-terminus (**Figure 9**).

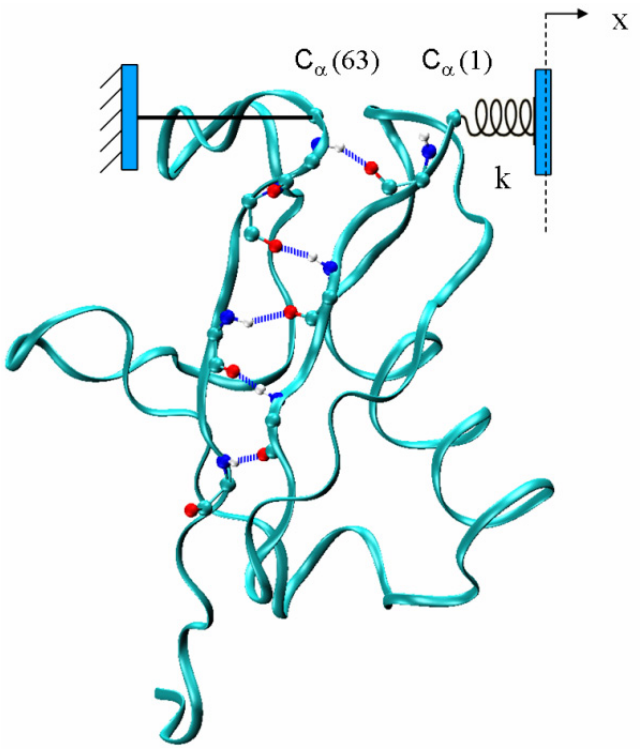

Fig. 9. PNW simulation scheme. The Cα of Lys63 was fixed and a harmonic potential was applied to the Cα of the N-terminal. The ubiquitin molecule was solvated in a box of 49.6 X 40.3 X 49.6 Å containing 2898 water molecules. Steps of 4 Å of extension and 100 ps of simulations were used.

Figure 10 shows the behavior of the elastic force of donor acceptor (**Figure 10**) distances between strands β1 and β5 during a long equilibration. The figure also shows a sequence of snapshots of the key events during the extension.

The H-bond from Gln 2(CO) to Glu 64(NH) breaks first (**Figure 11**), followed by the Phe 4(NH)-Ser 65(CO) H-bond, and so on in a zipper- like sequence along the entire segment.

In another simulation the Cα of the N-terminus was fixed and the Cα of Lys 48 was pulled with an external force (elastic constant of 3 kcal /mol Å^2) along the Cα to Cα axis, with 4 Å and 200 ps per step. The same five H- bonds between strands β1 and β5 break with a maximal force of 580 pN (results not shown).

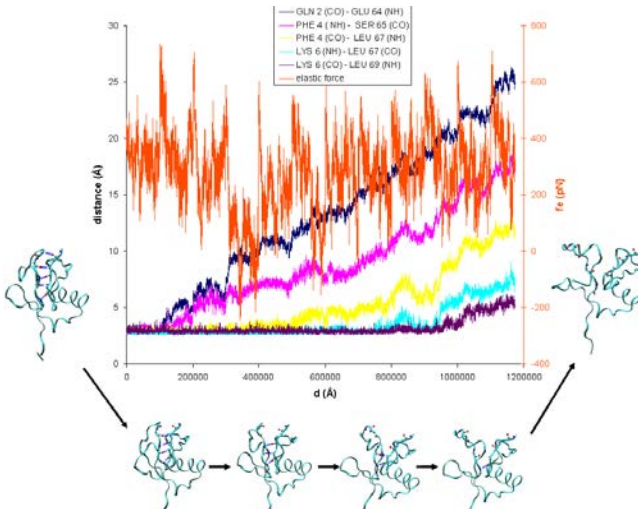

Fig. 10. Distances between H-bond donor and acceptor of parallel strands β1 and β5. The simulations show a zipper-like unraveling of individual backbone hydrogen bonds. The external elastic forces responsible for the H-bond breaking range between 200 to 600 pN.

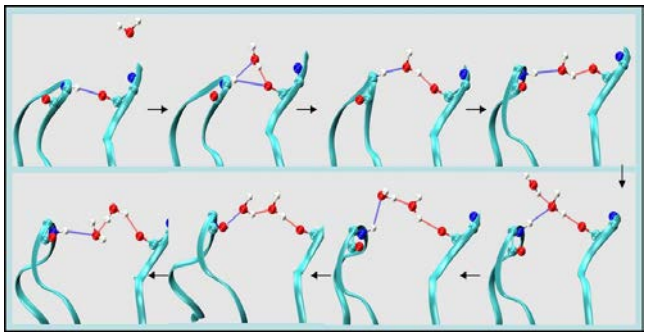

Fig. 11. Sequence of events leading to breaking of the GLN2 (CO)-GLU64 (NH) H-bond. Two water molecules are involved in the process.

Discussion

AFM studies of ubiquitin have exploited its unusual ability to form homopolyproteins in which stretching forces can be transmitted to various segments of the monomer via different linkages (different attachment points). Previous Steered Molecular-Dynamics (SMD) simulations of ubiquitin monomers in water and stretched by either of two linkages (17) qualitatively reproduced the differences between their mechanical unfolding patterns observed in AFM experiments, but the unfolding forces required in these simulations are at least an order of magnitude greater than those measured by experimental techniques. The PNW

simulation protocol (21) -short extensions followed by long equilibrations- corresponds more closely to the conditions of the experimental work. PNW is similar to constant velocity SMD stretching but with one main difference: the restraint point is not moved at constant speed. Instead, it is moved in short leaps that stretch the spring, after which the protein is allowed to equilibrate to the new situation and the simulation is continued for varied amounts of time to collect information about the force, the extension and the atomic details of the events that take place.

In SMD simulations, using either constant speed or constant force, the system is continually "catching-up" with the moving point and is never able to equilibrate with the new situation, which is continuously changing. This characteristic makes it difficult to observe detailed mechanistic information about the bond-breaking events that lead to the disruption of the native structure. In PNW, after an instantaneous extension, the system is allowed to relax for a period of time (typically 30-50 ps) and then observed during the following 100-200 ps. It is during this latter period that the most interesting details of the process, such as the mechanism of the participation of water molecules in the backbone H-bond separation, are observed.

Although simulations carried out by the PNW procedure show features of the unfolding process and force profiles similar to those reported by others (17), they show significant differences in the force peak value and in the details of the mechanism by which main-chain H-bonds break. Monitoring individual hydrogen bonds reveals that five hydrogen bonds between two parallel β-strands (**Figure 1**) break almost concurrently if the structure is pulled between the C-and the N-terminus (**Figure 3**), as described previously (17), but by a zipper-like sequence (**Figure 10**) if the pulling process is between N-terminus and Lys 63. A full analysis of the main chain H-bond behavior during the simulations shows that at low forces, before the force peak H-bonds remain mainly formed during the process, although they break and re-form frequently. In addition, water molecules make transient H-bonds to H-bonded main chain atoms, including cases in which one water molecule makes H-bonds with both the NH and the CO of a main chain H-bond pair. These bridging water molecules play a key role in the mechanism of unfolding: at forces closer to the peak force, once the same water molecule makes H-bonds to the donor and the acceptor of a main chain H-bond, the bond may break because the donor and the acceptor will remain hydrogen bonded, but now to the bridging water molecule. Under these conditions, the distance between the NH and the CO can extend beyond 3.5 Å, but remains below 4.0 Å, while making H-bonds to the single water molecule. Full separation of the strands requires that a second water molecule makes an H-bond to either the CO or the NH to allow the first water molecule to release one of the groups. Thus, water molecules do not replace backbone H-bonds after they break--they form hydrogen bonds to the backbone groups before the backbone H-bonds can break. This mechanism is shown explicitly in Figure 11, in which only after two water molecules bind one to the C=0 and the other to the NH of the backbone full separation of main-chain H-bonds is possible (34).

Conclusion

In summary, by using a simulation protocol that allows equilibration at each extension (PNW protocol) we have been able to observe the details of the events leading to the unfolding of ubiquitin by mechanical force. The breakage of the five H-bonds connecting the N-terminal and C-terminal β-strands corresponds to the main energy barrier separating the folded and unfolding states. Given adequate time, the applied force destabilizes these bonds with respect to the H-bonds the same backbone groups can make to water molecules, allowing the formation of stable H-bonds between single water molecules and the donor and acceptor groups of the interstrand H-bonds. Further separation requires that a second water molecule bind to the donor or the acceptor groups of each pair. This mechanism ensures that: a) no H-bond is broken before it is replaced; and b) the barrier for the separation of the folded and the unfolded states is in part kinetically determined and becomes larger as the speed of application of the force increases in agreement with experimental observations.

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